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Enhanced Xylanase Production from *Thermomyces lanuginosus* NCIM 1374/ DSM 28966 using Statistical Analysis

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Extracellular xylanase production by *Thermomyces lanuginosus* (NCIM-1374/ DSM 28966) could be enhanced using statistical tools of Response surface methodology. Optimized process parameters (pH: 6.3; temperature: 52°C; fermentation time: 108 h; substrate concentration: 1.8% wheat bran) were obtained using ANNOVA. Saccharification process under optimized condition enhanced extracellular xylanase production from 10012 IU/L/min to 12060 IU/L/min. Experimental design showed high correlation between predicted and experiment R-squared values, and analysis of variance had computed F-value of 16.57 with a very low P-value indicating the statistical significance of the quadratic model that can be used to navigate the design space with an adequate precision measure and could optimize the process for high level xylanase production. *Thermomyces lanuginosus* NCIM1374/ DSM 28966 is an indigenous strain isolated in our laboratory and its xylanase has unique capacity of hydrolyzing xylan to xylose. In the present study we were able to enhance extracellular production of this swift and differently acting enzyme.

Keywords: *Thermomyces lanuginosus*, xylanase, Response surface methodology, Quadratic model, Box- Behnken Design.

Hydrolysis of xylan (hemicellulose, second most abundant polymer in plant cell wall) is majorly catalyzed by xylanases followed by several other xylanolytic enzymes that includes â-d-xylosidase and a variety of debranching enzymes i.e. á-l-arabinofuranosidases, á-glucuronidases, acetyl esterases etc. [1-5]. Xylanase are produced by wide range of bacteria[6-8], fungi[9, 10], actinomycetes and yeast[11-13], among these fungi are significantly higher producers[14] and non cellulytic fungi, found in self heating masses of organic debris are major contributors. *T. lanuginosus* is one such non cellulytic fungi reported to produce cellulase free xylanases[15, 16].

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Xylanases find potential application in pulp and paper industries, baking industries, food and feed, breweries etc.[17-20] and are produced at large scale by submerged and solid state fermentation[21]. In order to reduce cost of production, agricultural wastes rich in xylan (wheat bran, corn cob, sugarcane Bagasse etc) are utilized as substrates1[7, 22-23] and all these processes are optimized by generic or computed statistical tool at laboratory scale. Response surface methodology is a statistical technique collectively used for designing experiments, plotting graphs and finally it also helps to evaluate the effect of various factors in the experiments and defines an optimal condition.

In the present report we have described optimization of process parameter for xylanase

production using sugarcane bagasse as substrate, by statistical analysis based on Box- Behnken Design of Design Expert Software by Response Surface Methodology[24, 25].

MATERIALS AND METHODS

Microorganism and fermentation conditions

Thermomyces lanuginosus (NCIM 1374/ DSM 28966) strain was isolated in our laboratory from soil sample containing plant wreckage material and maintained by growing on YPSs (yeast phosphate soluble starch agar) slants, incubated at 50°C for 5 days[26]. One milliliter of conidial suspension (10^5 conidia ml^{-1}) grown on Potato Dextrose Broth for 96 h were used as pre-inoculum of 100 ml production medium (1.5% Yeast extract and 0.5% KH_2PO_4, pH 6.5, 1.5% wheat bran) in 500 ml Erlenmeyer flasks[27, 28]. After saccharification process (varied time period based on experiment designed by Design Expert Software; with pH range 5.5-7.5; temperature 40°C- 60°C; fermentation time 72h to 148 h; substrate concentration (wheat bran) 5 gL^{-1} to 25 gL^{-1}) extracellular extract were used as enzyme samples for analysis. Samples were analyzed in triplicates and mean value taken. Enzyme activity was calculated by estimation of reducing sugar based on Dinitro-salicylic assay method[29] and one unit of enzyme activity was defined as millimoles of substrate hydrolyzed or product formed by reaction as specified temperature and pH condition in one minute of reaction.

Optimization of physicochemical parameters by response surface methodology

Four factor, three level Box-Benhken design was employed to investigate statistically the main and interactive effects of the four process variables selected for the study on production of xylanase. After investigation of 4 different variables ie, pH values (A), temperature (B), fermentation time (C) and substrate concentration (D), the nonlinear quadratic model[30] is given as equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j + \varepsilon \quad ...(1)$$

In the above equation, Y, k, $\hat{a}_0$, $\hat{a}_i$, $\hat{a}_{ii}$, $\hat{a}_{ij}$ and X_i are predicted response, number of factor variables, model constant, linear coefficient, quadratic coefficient, interaction co-efficient and factor variables in coded form respectively. The respective high low levels with coded levels in parentheses for variables corresponding to 5.5 (-1) and 7.5 (+1) for pH, 40°C (-1) and 60°C (+1) for temperature, 72 h (-1) and 168 h (+1) for incubation time and 0.5% (-1) and 2.5% (+1) for substrate concentration were used to design a total of 29 experiments with five replicates at the center point which allows estimation of a pure error sum of squares using Design Expert v7.0.3 (Stat-Ease, Inc., Minneapolis, USA) software. The response value in each trial was an average of triplicate runs.

RESULTS

Experimental design and its significance

Coefficient of variation (R^2= 0.9431) of the designed experiment indicated high correlation between experimentally observed and predicted values. The values of the predicted R^2 (0.7027) and the adjusted R^2 (0.8862) were within 0.20 of each other indicating a reasonable agreement between the experimental and predicted values of the xylanase activity and suggests the accuracy of model designed. Lack of fit was not significant indicating the model is fit for prediction and statistical significance of this model is given by the equations (Eq. 2) for coded factors. Predicted

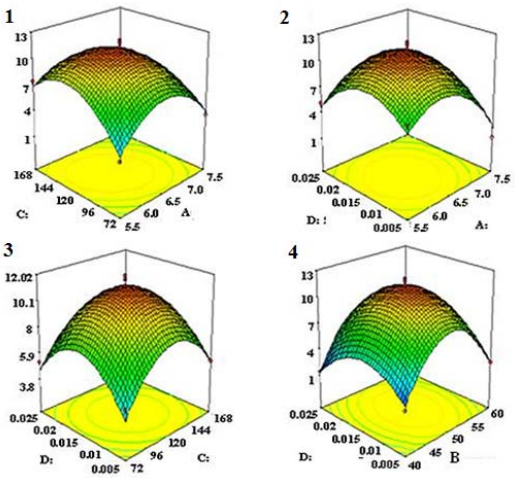

Fig. 1. Highly significant quadratic model for optimization of extracellular xylanase production by *Thermomyces lanuginosus* NCIM 1374/ DSM 28966 (A: pH value of medium; B: Temperature; C: Fermentation time; D: Substrate concentration)

response *Y* for high xylanase activity was obtained by the following second-order polynomial equation:

$$EA = 11.18 - 1.18A + 0.41B + 0.19C - 0.18D - 0.28AB - 1.46AC + 0.54AD - 0.20BC + 0.35BD - 0.46CD - 3.71A^2 - 5.68B^2 - 3.49C^2 - 3.28D^2 \quad \text{(Eq. 2)}$$

Where, EA, A, B, C and D are enzyme activity, pH, temperature, incubation time and media component respectively. ANOVA of quadratic regression model demonstrated that a computed *F*-value (16.57) and a very low *P*-value (0.0001; less than 0.05) showed significance of quadratic regression and model terms (A, AC, A^2, B^2, C^2, and D^2) respectively. The quadratic model designed could navigate the space with an adequate precision measure of 12.47.

Optimized process parameters

Maximal extracellular xylanase produced after optimization using single variable alterations was 10012 IU/L/min[23]. Xylanase production and its activity were affected by various parameters that are evident from the Contour plot given as Fig. 1. By taking statistical approach for getting optimized process parameters for enhanced xylanase production, total extracellular xylanase production was enhanced to 12060 IU/L/min. Optimized process parameters were pH: 6.3; temperature: 52°C; fermentation time: 108 h with 1.8% wheat bran as substrate.

DISCUSSION

Optimizing different set of parameters for any process using statistical tool is in high application since past few years and gives very promising results for the same [31-37]. In the present study a significant experimental design for establishing a standard protocol for enhanced extracellular xylanase expression was obtained and this result was comparable and in some cases better than other reports [38-43]. Temperature optimum for xylanase production by *T. lanuginosus* NCIM 1374/ DSM 28966 was analogous to those from other *Thermomyces* strains[44, 45], whereas fermentation time for the same was reduced comparatively. Xylanase from *T. lanuginosus* has been reported earlier for its novel mode of action[17, 23], results from the present study has helped to enhance extracellular xylanase production from 10012 to 12060 IU/L/min in reduced time and at decreased temperature. This stands beneficial as compared to various other studied microorganisms[46-49] and gives a broader commercial applicability to the xylanase under study at our laboratory. There are several studies reporting on various new aspects of enzyme engineering, modeling, novel platform technologies, and microbial interactions through systems biology which could be a better option for exploring the functionality of industrial enzymes[50-56]. Moreover, the future of industrial enzymes lies on few low temperature labile enzymes, functional proteomics and functional aspects of xylanases[57-59]. Nevertheless such technologies will improve the yield and productivity of any industrial enzymes.

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